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Cold-inducible RNA-binding protein (CIRP) regulates target mRNA stabilization in the mouse testis

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ABSTRACT

Cold-inducible RNA-binding protein (CIRP) is an RNA-binding protein that is expressed in normal testis and down-regulated after heat stress. Recent studies suggest that CIRP contributes to male fertility problems but the mechanisms are unclear. The purpose of this study was to identify the likely mechanism of CIRP in reproduction. Based on the RNA-Binding Protein Immunoprecipitation-Microarray (Chip) Profiling (RIP-Chip) and biotin pull-down assays, we found that the mRNAs binding with CIRP in testis were mostly associated with translation regulator activity, antioxidant activity, envelope and reproduction, including important mRNAs related to male infertility. We also discovered that (Un)($n \geqslant 2$) was the possible core recognition sequence, and the binding mRNAs increased their stabilization. Our results improve our understanding of the mechanism by which heat stress causes male infertility.

1. Introduction

Cold-inducible RNA-binding protein (CIRP, official gene name Cirbp) is a highly conserved glycine-rich RNA-binding protein, which contains an amino-terminal consensus sequence RNA-binding domain and a carboxyl-terminal glycine-rich domain [1,2]. Although the exact functions are unknown, it is thought that it modulates translation [3,4] and functions as RNA chaperones that facilitates upon the perception of cold stress [5,6]. CIRP was firstly discovered in the transcripts induced by DNA damage as a result of UV irradiation or UV mimetic agents [7,8]. CIRP was also described as hypoxia-responsive by an HIF-1-independent mechanism [9] and was considered as a stress-inducible protein that participates in the cellular response to oxidative stress inducers [10]. The protein was further identified as induced upon mild cold-shock [11,12]. In neural stem cells, moderate low temperature stimulates the expression of CIRP, which prevents neural stem cell from apoptosis [13]. CIRP plays an important role in the neuroprotective effects of hypothermia [14], including the blockage of cell apoptosis induced by TNF-alpha [15]. Recently, CIRP was described as a new generation of proto-oncogenes [10].

CIRP is constitutively expressed in testis, which is a heat sensitive organ. CIRP is down-regulated in the testis after heat stress caused by cryptorchidism, varicocele or other environmental heat stress. It is well described that the expression of CIRP decreases in male germ cells at elevated temperature [16], and over-expression of CIRP reduces testicular damage induced by experimental cryptorchidism [17]. Available data from other studies indicates that CIRP has a close relationship with hypoxia and oxidative stress and prevented cells from apoptosis induced by stress, which lead us to link down-regulated CIRP to male fertility problems caused by heat stress.

It is well known that spermatogenesis is a temperature-dependent process, and spermatogenic function is impaired after heat stress, and it is a complex of numerous pathological processes, including hypoxia and oxidative stress. Paul C demonstrated that transient mild hyperthermia causes hypoxia and oxidative stress and this occurs in a temperature-dependent manner [18]. Hypoxia has been shown to result in cell cycle arrest and apoptosis [19,20]. High levels of free radicals and reactive oxygen species (ROS) cause oxidative damage to DNA, and several studies have demonstrated that oxidative stress contributes to male fertility problems [21–23]. In many cell types, hypoxia and oxidative stress had been shown to trigger apoptosis and cell death [24,25]. Banks S reported that a mild scrotal heat stress had an impact on DNA integrity in murine spermatozoa [26], and the fragmentation of DNA was considered as a hallmark of apoptosis [27]. CIRP can respond to hypoxia and oxidative stress and reduce the damage caused by them, but CIRP is down-regulated after heat stress in testis which may result in serious damages to spermatogenic function, although the detailed mechanisms are unclear.

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In earlier studies, testis were subjected to heat stress to evaluate the functions of CIRP [16,17,26], there are no direct evidences to clarify the relationship of CIRP and spermatogenesis. In this study, we identify the target mRNAs bound to CIRP, validate potential recognition sequences, and reveal the biological effects of this binding, which are very helpful for revealing the mechanism of male infertility cause by heat stress.

2. Materials and methods

2.1. Animals

BALB/c mice (8-week-old) were purchased from the Experimental Animal Center of Wuhan University and were raised in the ABSL-3 laboratory of Wuhan University. All animal procedures used in this study conformed to NIH guidelines and were approved by the Ethics Committee of Wuhan University.

2.2. RNA-Binding Protein Immunoprecipitation assay

RNA-Binding Protein Immunoprecipitation (RIP) assay was employed to isolate target RNAs from CIRP. RIP assay was performed according to the kit protocol (Magna RIP™ RNA-binding Protein Immunoprecipitation Kit, Millipore, Billerica, MA, USA). Briefly, 100 mg testicular tissue was teased apart by Dounce homogenizer and re-suspended in complete RIP Lysis Buffer. Anti-CIRP antibody (MBL International Corporation, Woburn, MA, USA) and Normal Rabbit IgG (Millipore, Billerica, MA, USA) were used for RIP assays and defined as RIP-CIRP Group and RIP-IgG Group (negative control). The antibodies were incubated with Magnetic Beads Protein A/G for 30 min, the beads–antibody complexes were incubated with complete RIP Lysis Buffer at 4 °C for 10 h. Fifty microliter each out of 500 μl of the beads suspension during the last wash were removed to test the efficiency of immunoprecipitation by western blotting. Target RNAs were purified with procedures of proteinase K digestion and phenol–chloroform–isoamyl alcohol (125:24:1, pH 4–5) (Sigma–Aldrich, St. Louis, MO, USA) extraction, then precipitated in ethanol and re-suspended in 10 μl of RNAse-free water.

2.3. RNA amplification

The target RNAs isolated from CIRP were amplified and labeled by the procedure of In vitro transcription (IVT). According to the kit protocol (MessageAmp™ II-Biotin Enhanced Kit, Single Round aRNA Amplification Kit, Ambion, Grand Island, NY, USA), reverse transcription of the target RNAs were performed to synthesize first strand cDNA with T7 Oligo(dT) Primer. Second strand cDNA was synthesized with DNA polymerase and purified with cDNA Filter Cartridge. In vitro transcription was started by adding T7 Enzyme Mix to the system with the presence of Biotin-NTP Mix. Biotin labeled antisense amplified RNA (aRNA) was purified by aRNA Filter Cartridge, and fragmented for microarray analysis.

2.4. Microarray assay

Affymetrix GeneChip® Mouse Genome 430 2.0 Array was employed to analysis the species of aRNA, which produced by Shanghai Biochip Corporation (SBC, Shanghai, China). Array hybridization and wash were performed using GeneChip® Hybridization, Wash and Stain Kit (Affymetrix, Santa Clara, CA, USA) in Hybridization Oven 645 (Affymetrix, Santa Clara, CA, USA) and Fluidics Station 450 (Affymetrix, Santa Clara, CA, USA) followed the manufacturer's instructions. Slides were scanned by GeneChip® Scanner 3000 (Affymetrix, Santa Clara, CA, USA) and Command Console Software 3.1 (Affymetrix, Santa Clara, CA, USA) with default settings. Raw data were normalized by MAS 5.0 algorithm, Gene Spring Software 11.0 (Agilent technologies, Santa Clara, CA, USA). All data were analyzed with SBC Analysis System (SBC, Shanghai, China). The top 1000 abundant mRNAs were selected for functional annotation.

2.5. RT-PCR

Twenty-four mRNAs out of 66 mRNAs annotated as reproduction were selected randomly and validated by RT-PCR. The primers are shown in Table 1. Target mRNAs were isolated from RIP assay, including RIP-CIRP group (C), RIP-IgG group (I) and total RNA (T). RNAs were reverse-transcribed to cDNA and detected by PCR according to manufacturer's protocol (TaKaRa, Otsu, Shiga, Japan).

2.6. Biotin pull-down assays

Prm2 mRNA was taken as the model for biotin pull-down assays. Prm2 mRNA showed high normalized signal value (NS value) in microarray data and validated by RT-PCR, and the lengths of 5′Untranslated Region (5′UTR), Coding Region (CR) and 3′Untranslated Region (3′UTR) were suitable for fragmental PCR. Total RNA was reverse-transcribed to cDNA for PCR amplification by High Fidelity PrimeScript® RT-PCR Kit (TaKaRa, Otsu, Shiga, Japan), the forward primers contained T7 RNA polymerase promoter sequence: 5′-CCAAGCTTCTAATACGACTCACTATAGGGAGA-3′. Primers used for the preparation of biotinylated transcripts spanning Prm2 mRNA 5′UTR, CR and 3′UTR are shown in Table 2. The PCR amplified fragments were purified and used as templates for IVT by T7 Enzyme Mix in the presence of Biotin-NTP Mix or NTP Mix (negative control group). Six microgram transcripts were incubated with 120 μg of testis tissue lysates for 30 min at room temperature [28]. The complexes were isolated by Hydrophilic Streptavidin Magnetic Beads (New England BioLabs, Ipswich, MA, USA), the affinity of different fragments with CIRP were evaluated by detecting the levels of CIRP in pull-down materials by Western blotting.

2.7. mRNA mutation

According to the prior work done by Nishiyama [12] and our results of biotin pull-down assays, we predicted that UU or UUU or more repeat U sequence was the possible recognition sequence. UU and UUU were inserted into Prm2 mRNA CR fragment, which showed low affinity with CIRP in the biotin pull-down assays. The primers used are shown in Table 3. AA or AAA was added before restriction enzyme cutting site of BamHI in reverse primer. The PCR products were from high fidelity RT-PCR system and purified by DNA Gel Extraction System. Fragments were incubated with BamHI enzyme at 30 °C for 1 h. T4 DNA Ligase was employed for the ligation reaction after the purification of the fragments. The products were purified for sequencing and IVT. Biotin pull-down assays were performed to analyze the affinity of the mutated fragments with CIRP.

2.8. Transient transfection of siRNA

The sequences of siRNAs and negative control sequences are shown in Table 4, both of them were designed on the website of Dharmacon.com and synthesized by Dharmacon (Chicago, IL, USA) with chemical modification including 2′-Ome, phosphorylation and cholesterol modification. Normal testis was used as a control group and nonsense siRNA complex was used as negative control group (NC group). About 10 μg siRNA complex was diluted in 20 μl PBS and incubated with 30 μl Lipofectamine™ 2000 reagent (invitrogen, Grand Island, NY, USA) at room temperature

Table 1
Primers for RT-PCR.

Name	Accession No.	Forward primer	Reverse primer	Product length
Adad1	NM_009350.3	AGGACTGGGACAGAATAA	TCTTGGCATACTGGACTT	203
Psme4	NM_134013.3	AGCCTATTGAGATGACTGT	CCTGTGAGCGAAGACTAA	304
Agfg1	NM_010472.2	AGTTAGACAGCGTGTTCAG	GATTCGTAGAAGGCGTAG	165
Ddx25	NM_013932.4	TGAACTTTGACCTCCCTGTA	TCTCACGTAAACTAGCCCAC	273
Mael	NM_175296.4	GGTGTTTGAAGCGTATGG	CGTGTTGCTAGAATAGTCCC	170
Spag6	NM_015773.2	TTGCTCACTTAGCCCAGAT	CAGCCGTATGTTCCCTTT	302
Tnp1	NM_009407.2	CAGCCGCAAGCTAAAGAC	AAGACCACCAGGGCAGAG	190
Klhl10	NM_025727.3	ACTTCACCAACCCACTTACC	CATTCACCCATCTGTCTGC	129
Pebp1	NM_018858.2	TGCCAGCCGTCACGATTC	CTTGATACCTACGCAGCCAC	326
Gpx4	NM_001037741.2	GGCAGGAGCCAGGAAGTAATC	CACCACGCAGCCGTTCTTAT	212
Odf4	NM_145746.2	AGCAGCACCACCATCAAC	GCCACAGACAAAGCCAAG	260
Hspa1 l	NM_013558.2	CACCAAGCAGACGCAGAT	AGCCAGGAAAGGACCTCA	480
Mea1	NM_010787.1	GGGTGAAGGAGAAGATGG	GGAAGGAGGAGGCTGTTA	389
Catsper1	NM_139301.2	AGAGTGGACCTCGTATTGACC	TCCGTGATAGTGCCGTTG	335
Zfp541	NM_001099277.1	ACCCACCGTCCAACTCCT	GGGCATTCCGACTCTTGAT	187
Fscn3	NM_019569.2	GACACCTTCTTCCGAGTGC	CCCTGTGCCTGAAAGTGATAG	293
Tcp11	NM_013687.3	TGCCTCTGTCTTGCTGGTG	AGGGACGCCGTATTCTCG	225
Hils1	NM_018792.1	CACAGCCCACTCACTACCG	CAGCCAAAGTCTTCAGAATCAC	283
Prm2	NM_008933.1	ACAAGAGGCGTCGGTCAT	TTGGCTCCAGGCAGAATG	169
Csda	NM_139117.2	GCAAGCGGCTAATGGTCC	CTCGGCACTGCTCTGTTCG	175
Ggnbp1	NM_027544.2	GTGCCTCCACCTACCAGTCG	CAGTTTCCCATCTCGTCCATC	235
Prm1	NM_013637.4	CGCAGCAAAAGCAGGAGCAGAT	CTCACGCAGGAGTTTTGATGGA	174
Odf3	NM_027019.3	GCCCTGCCTACTCCATCCT	GCTCCTGGTCCTGGCTTCA	409
Tssk6	NM_032004.1	CCCTACGACCCTAAGAAATACGACG	ACGGGCAGACGGGCTGAACT	207

Table 2
Primers for fragmental PCR with T7 RNA polymerase promoter sequence. T7 RNA polymerase promoter sequence: 5′-CCAAGCTTCTAATACGACTCACTATAGGGAGA-3′.

Fragment	Forward Primer	Reverse Primer	Spanning site	Product Length
5' UTR	T7-ATCATCACCACCAAGAGC	TGGTGCCAGGAGATCAGG	1-100	100
CR	T7-TCGCTACCGAATGAGGAG	GGGAGGCTTAGTGATGGT	105-430	326
3'UTR	T7-CAGGAAATGTAGGAGGCA	CTCGTGTCAAGCTTTATT	396-612	217
3'UTRa	T7-CAGGAAATGTAGGAGGCA	CCTCACATGATGTTGCTT	396-510	115
3'UTRb	T7-ACCACCATTCCATGTCGA	CTCGTGTCAAGCTTTATT	515-612	98

Table 3
Primers for Prm2 CR fragment mutation. T7 RNA polymerase promoter sequence: 5′-CCAAGCTTCTAATACGACTCACTATAGGGAGA-3′. BamHI recognition site: GGATCC.

Fragment	Forward Primer	Reverse Primer	Spanning site
Fragment before BamHI (UU)	T7-TCGCTACCGAATGAGGAG	CGGGATCCAATATGTAGCCTCTTACGAG	105-283
Fragment before BamHI (UUU)	T7-TCGCTACCGAATGAGGAG	CGGGATCCAAATATGTAGCCTCTTACGAG	105-283
Fragment after BamHI	CGGGATCCACAAGAGGCGTC	GGGAGGCTTAGTGATGGT	284-430

Table 4
siRNA sequences for CIRP.

Name	Sense sequence	Antisense sequence
siRNA 1	GAGACAGCUAUGACAGUUAUU	UAACUGUCAUAGCUGUCUCUU
siRNA 2	GUGGUAAAGGACAGGGAGAUU	UCUCCCUGUCCUUUACCACUU
NC 1	GCAUAAGUUGAUGACAGCAUU	UGCUGUCAUCAACUUAUGCUU
NC 2	GAUAGAGAGAGAGGCGUGAUU	UCACGCCUCUCUCUCUAUCUU

for 20 min. Thirty microliter complex was transfected into seminiferous tubules though the method of microinjection described by Ogawa [29]. Thirty microliter Actinomycin D (200 μg/ml) (Sigma–Aldrich, St. Louis, MO, USA) was injected into seminiferous tubules in microinjected mice after 48 h. Testes were harvested in 0 h, 3 h and 6 h after Actinomycin D was injected. All of the testes were divided into two parts, one for knockdown validation by western blotting, and the other for mRNA stability analysis.

2.9. Western blotting

Tissues were lysed in triple-detergent RIPA buffer with protease inhibitor cocktail. Protein quantity was determined by BCA method. About 40 μg of protein was electrophoresed on SDS–PAGE and transferred to a PVDF membrane. The membrane was blocked with 5% non-fat milk at room temperature for 2 h and incubated with anti-CIRP antibody (1:1000) (ProteinTech Group, Chicago, IL, USA) and anti-β-Actin antibody (1:1000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4 °C overnight. Following secondary antibody incubations, signals were visualized by enhanced chemiluminescence.

2.10. mRNA stability analysis (quantitative real-time PCR)

mRNA stability was evaluated by detecting the residual mRNAs after the injection of Actinomycin D in groups. Csda mRNA was taken as the model for mRNA stability analysis because of the high NS value showed in microarray data and validated by RT-PCR. Total RNA was extracted from control group, negative control group and validated knocked-down samples using TRIzol reagent (invitrogen, Grand Island, NY, USA) according to manufacturer's protocol. Reverse transcription of total RNA (500 μg) was performed to synthesize cDNA with random hexamers primer according to the kit protocol (PrimeScript® RT reagent Kit (Perfect Real Time), TaKaRa, Otsu, Shiga, Japan). 18S rRNA was taken as a reference gene. The levels of Csda and 18S rRNA were detected by quantitative real-time PCR (SYBR® Premix Ex Taq™ II (Perfect Real Time), TaKaRa, Otsu, Shiga, Japan) in all groups. Csda Forward Primer: 5′-GCAAGCGGCTAATGGTCC-3′, Reverse Primer: 5′-CTCGGCACTGCTCTGTTCG-3′. 18S rRNA Forward Primer: 5′-AGAAACGGCTACCACATCC-3′, Reverse Primer: 5′-CACCAGACTTGCCCTCCA-3′. The Ct values of Csda in different groups were normalized by 18S rRNA and the differences were analyzed by relative quantification method ($2^{-\Delta\Delta Ct}$ method).

2.11. Statistical analysis

Statistical analyses were performed using SPSS software 13.0, all data were presented with mean (±) standard deviations (SD). One-way ANOVA was used for statistical significance analysis. Differences were considered significant if $p < 0.05$.

3. Results

3.1. RIP assay followed by aRNA amplification was proved to be an efficient method for microarray assay

RNA-Binding Protein Immunoprecipitation-Microarray (Chip) Profiling (RIP-Chip) is a new approach to identify the species of target mRNAs binding with RBPs. Quality control report for hybridization showed that the aRNAs were integrated (Fig. 1A) and abundant suggested that RIP assay followed by aRNA amplification was an efficient method for microarray assay. In the efficiency tests of RIP assays, CIRP appeared in experiment group but failed to be detected in negative control group (Fig. 1B) demonstrated that RIP assay isolated CIRP successfully, which is the foundation for the following assays. RIP assay had isolated about 100 ng RNA in RIP-CIRP group in the last step, but in RIP-IgG group, target mRNAs were not detected due to the low amount. Compared with the original templates, the abundance of RNA increased drastically after IVT and showed a statistically significant difference (RIP-CIRP group $p < 0.01$, RIP-IgG group $p < 0.05$). 28.9 μg and 4.7 μg biotin labeled aRNA were yielded in RIP-CIRP group and RIP-IgG group, which showed a significant difference ($p < 0.01$) (Fig. 1C).

3.2. The target mRNAs of CIRP in mouse testis are mostly associated with translation regulator activity, antioxidant activity, envelope and reproduction

Microarray data showed that there were 18021 positive mRNAs on the chip. Functional annotation for the top 1000 abundant mRNAs revealed that most of them were associated with translation regulator activity, antioxidant activity, envelope and reproduction (Fig. 2). Sixty-six mRNAs related to reproduction were presented in the annotation data, the NS values and accession numbers are shown in Table 5. Twenty-four mRNAs out of these mRNAs were validated by RT-PCR, the results showed that all of them appeared in

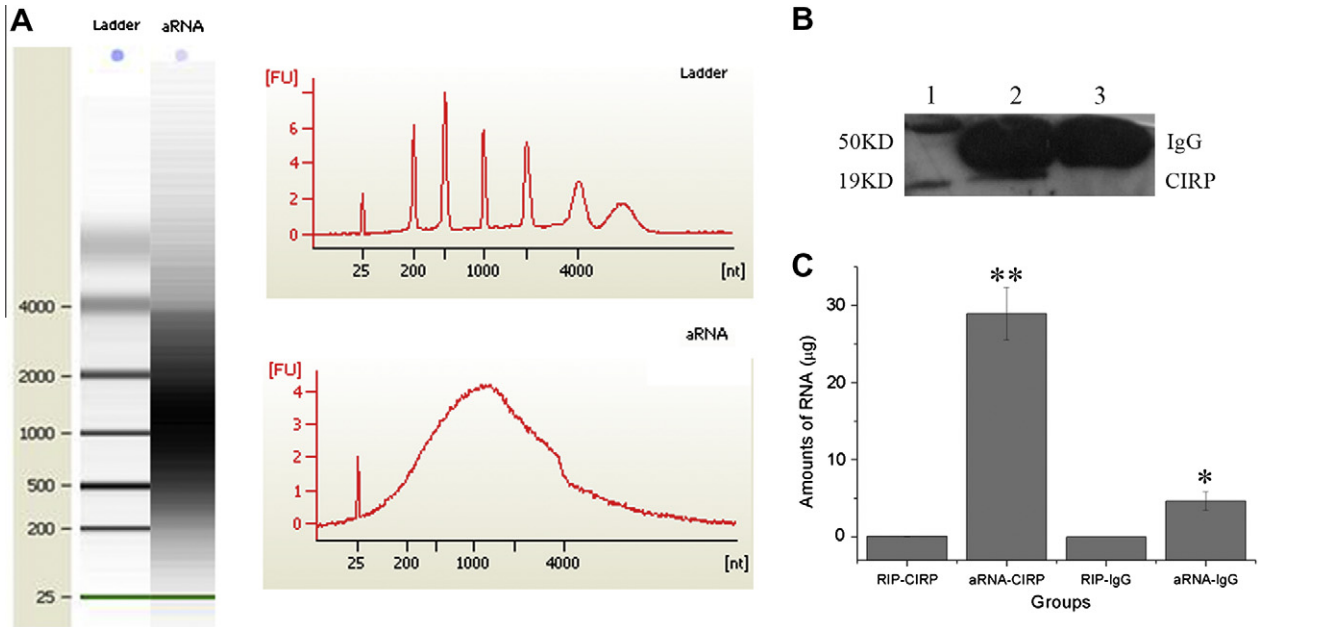

Fig. 1. The efficiency analysis of RIP assay for microarray. (A) Electrophoretograms of aRNA. Most of the aRNA spanned 500–2000 nt with low degradation. (B) The efficiency analysis of RIP assay. CIRP appeared in Input group (lane 1) and RIP-CIRP group (lane 2) and did not appear in RIP-IgG group (lane 3), which indicated that CIRP was specifically isolated by RIP assay. There was a nonspecific band in input group. (C) Bar graph of the RNA amplification. About 28.9 μg and 4.7 μg aRNA were yielded from IVT in RIP-CIRP group and RIP-IgG group respectively, which showed significant differences ($p < 0.01$). ($n = 3$).

GoTerm To Gene
Total input gene quantity: 1000
Total gene quantity of current species: 18021
Show Level: 1 2 3 pvalue <= Set Filter Cancel Filter

GOId	Name	Hits	Total	Percent	Enrichment test pvalue	qvalue
GO:0045182	translation regulator activity	22	100	22.0%	0.0	0.0
GO:0016209	antioxidant activity	6	34	17.65%	0.0173	0.112
GO:0031975	envelope	61	464	13.15%	0.0	0.0
GO:0000003	reproduction	66	531	12.43%	0.0	0.0
GO:0022414	reproductive process	65	528	12.31%	0.0	0.0
GO:0005198	structural molecule activity	37	374	9.89%	0.0010	0.011
GO:0032991	macromolecular complex	167	1901	8.78%	0.0	0.0
GO:0044422	organelle part	188	2346	8.01%	0.0	0.0
GO:0043226	organelle	512	7917	6.47%	0.0	1.0E-4
GO:0031974	membrane-enclosed lumen	39	612	6.37%	0.2193	0.7725
GO:0003824	catalytic activity	298	4816	6.19%	0.0187	0.1182
GO:0010926	anatomical structure formation	46	771	5.97%	0.3349	0.875
GO:0008152	metabolic process	415	7083	5.86%	0.0888	0.4152
GO:0051234	establishment of localization	127	2358	5.39%	0.652	0.875
GO:0051179	localization	148	2744	5.39%	0.6566	0.875
GO:0030234	enzyme regulator activity	33	622	5.31%	0.6266	0.875

Fig. 2. Results of microarray and GoTerm to Gene analysis. There were 18021 mRNAs presented in microarray data. The top 1000 abundant mRNAs were selected for functional annotation. The most abundant mRNAs were associated with translation regulator activity, antioxidant activity, envelope and reproduction. Sixty-six mRNAs related to reproduction were presented in the annotation data.

Table 5
mRNAs related to reproduction in the annotation data.

mRNA	NS value	Accession number	mRNA	NS value	Accession number
Tdrd7	13.6721	NM_146142.1	Agpat6	13.7099	NM_018743.4
Psme4	13.991	NM_134013.3	Kdm3a	12.7446	NM_173001.2
Ehmt2	13.6521	NM_145830.1	Acr	12.9628	NM_013455.3
Arsa	13.1679	NM_009713.4	Ccna1	12.7782	NM_007628.3
Nr6a1	13.512	NM_010264.4	Gykl1	13.6806	NM_010293.3
Hexa	12.8929	NM_010421.4	Agfg1	13.0121	NM_010472.2
Hspa1l	13.8923	NM_013558.2	Smcp	13.7265	NM_008574.3
Mea1	14.0207	NM_010787.1	Mast2	12.8008	NM_001042743.1
Odf1	14.39	NM_008757.3	Odf2	13.7817	NM_001113213.1
Pafah1b1	13.5	NM_013625.4	Pafah1b2	13.274	NM_008775.3
Plcd4	12.9144	NM_001081456.1	Prm1	14.2479	NM_013637.4
Prm2	14.6808	NM_008933.1	Prm3	12.9403	NM_013638.2
Rps6	13.3129	NM_009096.3	Sod1	12.7959	NM_011434.1
Spa17	13.0461	NM_011449.2	Spin1	12.8815	NM_011462.2
Nhp2l1	13.6414	NM_011482.4	Tcp11	14.2592	NM_013687.3
Adad1	13.6579	NM_009350.3	Tnp1	14.3294	NM_009407.2
Tnp2	14.2229	NM_013694.4	Tssk1	12.8152	NM_009435.2
Tssk2	13.8249	NM_009436.2	Slc26a8	13.0178	XM_003085148.1
Catsper1	13.5852	NM_139301.2	Sf1	14.356	NM_001110791.1
Txnrd3	14.531	NM_001178058.1	Tbpl1	13.9457	NM_011603.5
Pebp1	14.7691	NM_018858.2	Odf4	13.2507	NM_145746.2
Adam1a	14.0492	NM_172126.2	Trim36	14.1618	NM_178872.4
Ddx25	13.4684	NM_013932.4	Spag6	13.0535	NM_015773.2
Zpbp	13.5363	NM_015785.2	Hils1	13.7936	NM_018792.1
Fscn3	13.2554	NM_019569.2	Csda	14.5624	NM_139117.2
Pvrl3	13.7079	NM_021495.4	Gpx4	13.8612	NM_001037741.2
Tbata	13.2329	NM_001017433.2	Zfp541	13.6055	NM_001099277.1
Klhl10	13.589	NM_025727.3	Atp8b3	14.5499	NM_026094.3
Spata6	12.9999	NM_026470.3	Odf3	13.8304	NM_027019.3
Zpbp2	12.9213	NM_027061.2	Ggnbp1	13.8468	NM_027544.2
Spata24	13.0556	NM_027733.5	Paqr5	13.2377	NM_028748.2
Spata19	12.8546	NM_029299.2	Herpud2	13.0567	NM_020586.2
Tssk6	13.7639	NM_032004.1	Mael	14.3084	NM_175296.4

RIP-CIRP group (C) and total RNA group (T), but undetectable in the RIP-IgG group (I) (Fig. 3). The results suggested that the microarray data are credible in these 66 mRNAs, and indicated that CIRP is associated with the mRNAs related to translation regulator activity, antioxidant activity, envelope and reproduction in mouse testis.

3.3. U-rich sequences were validated as possible core recognition sequences of CIRP in testis

The recognition sequence was evaluated by affinity analysis of different fragments of Prm2 mRNA with CIRP in vitro with biotin pull-

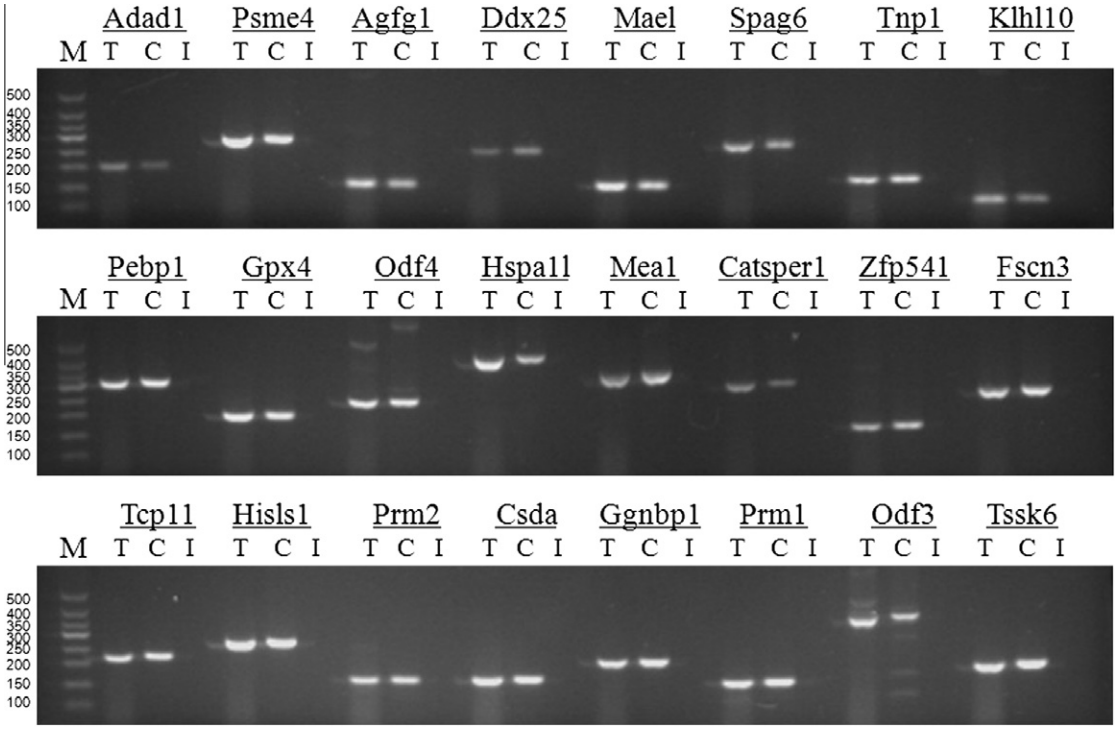

Fig. 3. The validation of the mRNAs associated with reproduction. All of the 24 mRNAs were detected in total RNA group (T) and RIP-CIRP group(C), but failed to be detected in RIP-IgG group (I).

down assays. Results showed that Prm2 mRNA binding with CIRP in the lysates in vitro. To seek for the recognition sequence, biotin labeled fragments of Prm2 were tested by pull-down assays (Fig. 4A), and the results suggested that 5′UTR, 3′UTR had high affinity with CIRP, while the CR fragment showed poor binding ability with it (Fig. 4B), which showed a significant difference ($p < 0.01$) (Fig. 4E). In addition, 3′UTR was divided into two parts (3′UTRa and 3′UTRb) for affinity analysis and both of them showed high binding ability with CIRP (Fig. 4C). No signal were detected in negative groups suggested there was no nonspecific binding of CIRP in biotin pull-down assay system. After analyzed the features of all the fragments and reviewed the existing findings from other groups [12,30], we made a prediction that UU or UUU or more repeat U sequence is the possible stable recognition sequence. To confirm our prediction, we successfully inserted UU and UUU to CR fragment (data not shown) which showed poor binding ability to CIRP, and the results indicated that the inserted sequences were conferred increased binding ability with CIRP (Fig. 4D). The differences between mutated sequences and the CR fragment were significant ($p < 0.01$) (Fig. 4F). All of the biotin pull-down assays indicated that (Un)($n \geqslant 2$) is the possible core recognition sequence of CIRP (Fig. 4G).

3.4. CIRP is associated with stability of target mRNAs

Compared with the control group and negative control group, CIRP was down-regulated in siRNA groups (Fig. 5A) and the differences showed significant statistical differences ($p < 0.01$). The differences between the negative control group and control group were not significant ($p > 0.05$) (Fig. 5B). The efficiency analysis of RNA interference showed that CIRP was successfully knocked-down in 0 h, 3 h and 6 h groups. mRNA stability analysis was performed in these validated knocked-down samples.

The levels of mRNAs were detected by quantitative real-time PCR and evaluated by Ct values. The larger CT value appeared, the less mRNA remained. Csda mRNA and 18S rRNA had similar amplification efficiency (data not shown) suggesting that the quantitative real-time PCR system was reliable. ΔCt values and fold changes in different groups are shown in Table 6. We found that the ΔCt values of Csda mRNA in control group and negative control group were similar and the difference had no significance ($p > 0.05$). Compared with control groups, the ΔCt values of Csda mRNA in 3 h and 6 h groups increased and showed significant differences respectively ($p < 0.01$). ΔCt values of Csda mRNA in 0hr group increased slightly but the difference had no significance ($p > 0.05$) (Fig. 5C), these maybe a result of endogenous transcription. Compared with control group, the levels of Csda mRNA in siRNA groups decreased to 0.834-fold, 0.353-fold and 0.255-fold respectively. The result showed that Csda mRNA degraded more serious in CIRP knocked-down mice and we can make a conclusion that mRNAs binding with CIRP increased their stability, and made them have a longer survival time.

4. Discussion

In this study, we found that there were 18021 species of mRNAs bound to CIRP in the mouse testis, and functional annotation suggested that most of them were associated with translation regulator activity, antioxidant activity, envelope and reproduction. We discovered that the mRNAs bound to CIRP increased their stability. We also validated UU and UUU were the possible core recognition sequences of CIRP. We firstly combined RNA mutation process with biotin pull-down assays in recognition site analysis, and it was proved to be a reliable method to validate the predicted sequence, especially for short sequence.

Many RNA-binding proteins (RBPs) have been identified in the testis and genetic studies showed they play important roles in spermatogenesis [31–34]. RBPs were proved to be related with RNA metabolism and regulate the temporal, spatial and functional dynamics of RNAs [35]. Recently, they were discovered to be associated with post-transcriptional regulation in gene expression

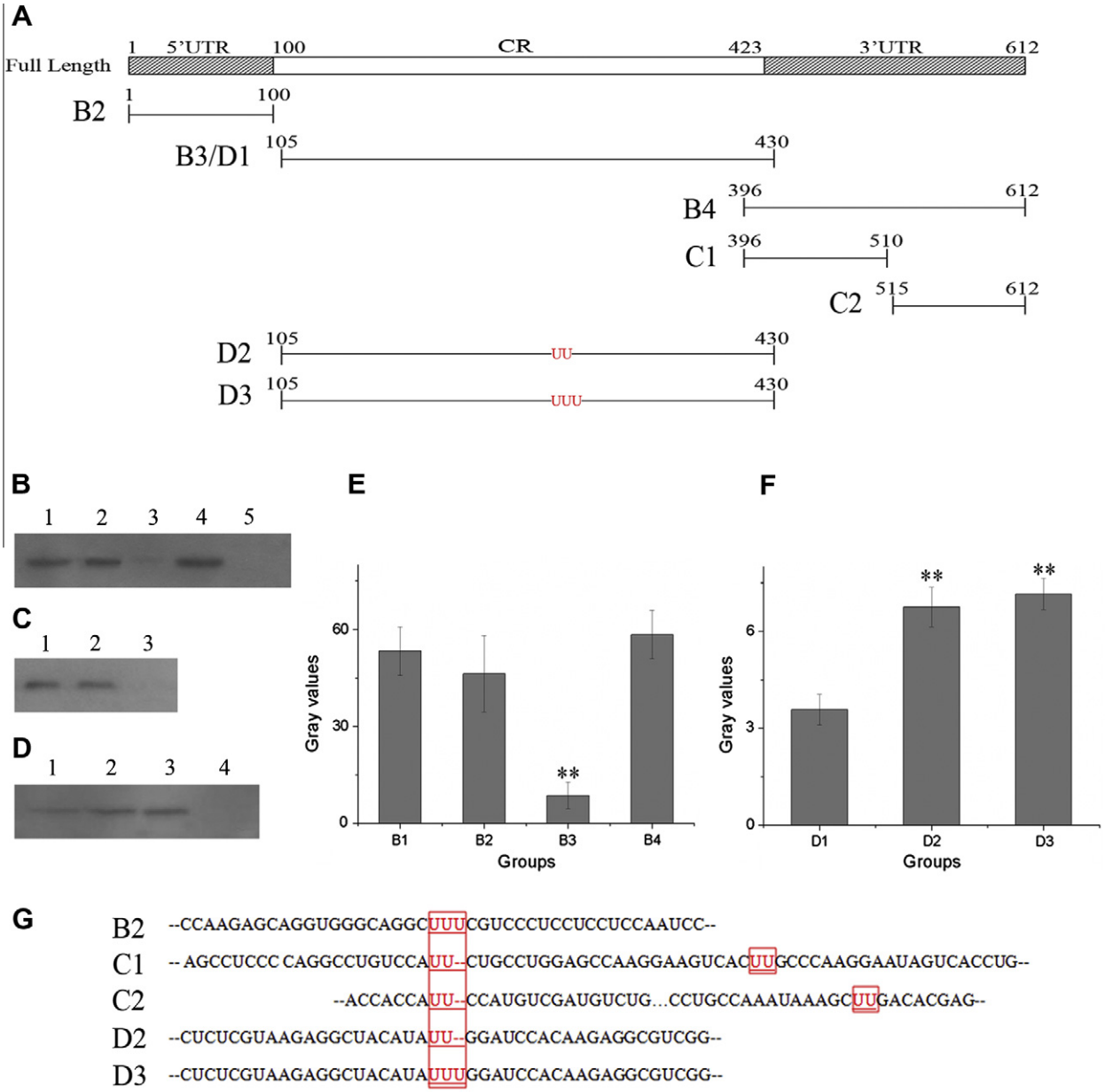

Fig. 4. Analysis of possible recognition sequence. (A) Maps for different fragments of Prm2 mRNA. (B) The affinity analysis of 5′UTR, CR and 3′UTR of Prm2 mRNA with CIRP. Biotin pull-down assay showed that the full length (lane 1), 5′UTR (lane 2) and 3′UTR (lane 4) fragments had high affinity with CIRP, but CR (lane 3) showed poor affinity with CIRP. No signal was detected in negative control group (lane 5). (C) The affinity analysis of the two parts of 3′UTR fragment with CIRP. The results suggested that both of the fragments (lane 1 and lane 2) can bind with CIRP. No signal was detected in negative control group (lane 3). (D) Mutated RNA fragments showed increased affinity with CIRP. Prm2 mRNA CR fragment showed poor affinity with CIRP (lane 1), and mutated RNA fragments with UU (lane 2) and UUU (lane 3) showed increased affinity. No signal was detected in negative control group (lane 4). (E) Bar graph of the gray values in full length, 5′UTR, CR and 3′UTR fragments. Gray values in full length, 5′UTR and 3′UTR showed no significant differences ($p > 0.05$), while it was decreased significantly in CR fragment ($p < 0.01$). (n = 3) (E) Bar graph of the gray values in CR and mutated RNA fragments. Compared with CR fragment, mutated RNA fragments with UU and UUU showed increased gray values, and the differences showed statistical significances ($p < 0.01$). (n = 3) (G) Consensus sequence analysis in all validated binding fragments.

[36–38]. In other cell types CIRP stimulates general protein synthesis and promotes proliferation. In embryonic stem cells, overexpression of CIRP increases the level of p-ERK1/2 and affects the phosphorylation of proteins such as 4EB-P1 and S6, which are involved in the initiation of translation [39]. Our findings are novel supplements for previous studies focus on CIRP in post-transcriptional regulation of gene expression. We found that the target mRNAs bound to CIRP increased their stability and made them have a longer survival time, which may be a novel mechanism of CIRP for the promotion of protein synthesis. Target mRNAs were mostly associated with translation regulator activity, antioxidant activity, envelope and reproduction in testis, from which we can infer that CIRP promote the expressions of those proteins, which is very important for the proliferation of spermatogenic cells in testis.

A number of azoospermia factor (AZF) mRNAs, such as DDX3Y, EIF1AY, HSFY2, RBMY1, RPS4Y2 and so on, are the target mRNAs bound to CIRP with relative high NS values. AZF proteins play important roles in spermatogenesis in different stages and the function deficiency of these proteins may cause male infertility [40]. In Agfg1 knocked-out mice, the sperm count decreases to 1/15 of the wild type mice. In Tbpl1 knocked-out mice, spermiogenic arrest at step 7 and no sperm is observed in seminiferous tubes [41]. Down-regulated CIRP may result in the reduced survival time

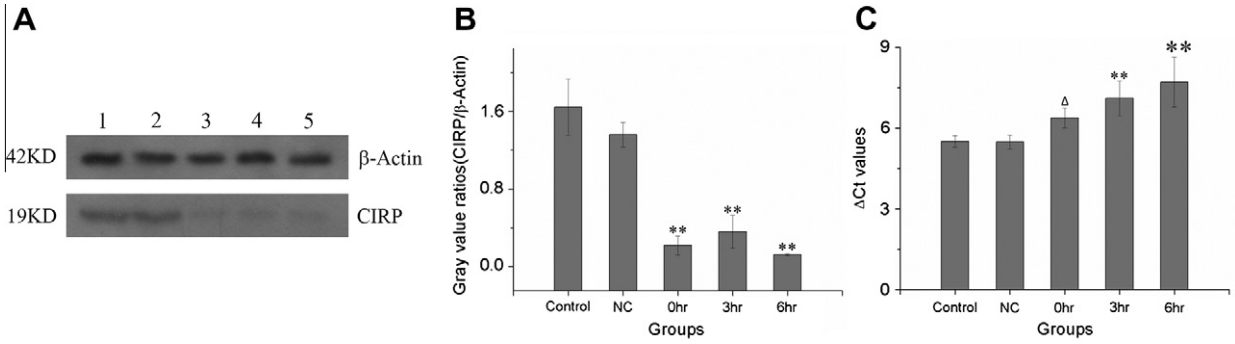

Fig. 5. Analysis of mRNA stability. (A) The efficiency analysis of RNA interference. Compared to the control group (lane 1) and negative control group (lane 2), CIRP was down-regulated by siRNA treatment (lanes 3, 4, 5). (B) Bar graph for RNA interference efficiency analysis. The results showed that the difference between control group and negative control group had no statistical significance ($p > 0.05$). Compared with control groups, gray values in siRNA groups decreased significantly ($p < 0.01$). ($n = 3$) (C) Bar graph of ΔCt values in different groups. The results showed that the difference of ΔCt values between control group and negative control group had no statistical significance ($p > 0.05$). Compared with control groups, ΔCt values in 3hr and 6hr groups increased significantly ($p < 0.01$), while the difference between 0hr and control groups had no significance ($p > 0.05$). ($n = 3$).

Table 6
ΔCt and Fold changes in different groups.

Groups	ΔCt	Fold changes
Control	5.503 ± 0.209	1
NC	5.487 ± 0.254	1.019 ± 0.157
0hr	6.377 ± 0.371	0.834 ± 0.350
3hr	7.106 ± 0.645	0.353 ± 0.097
6hr	7.709 ± 0.924	0.255 ± 0.115

of the target mRNAs including these mRNAs that crucial for spermatogenesis, which may be another mechanism of male infertility cause by heat stress in testis.

De Leeuw F reported that CIRP migrates from the nucleus to cytoplasmic stress granules by a methylation-dependent mechanism and acts as a translational repressor in response to stress conditions in Cos, Hela, NIH3T3 and 293T cells [42], which are different from previous results. The different subcellular localization and chemical modification of CIRP in different cells or tissues may have different functions [43]. CIRP is expressed in primary spermatocyte, secondary spermatocyte and round spermatid, but is not expressed in spermatogonia, elongating spermatids, Sertoli cells or Leydig cells. In primary spermatocyte, CIRP mainly appears in the nucleus but it is cytoplasmic in round spermatid [16]. The different subcellular localization of CIRP in testis indicates it may be a multifunctional protein in testis. It may have different physiological functions in primary spermatocytes and round spermatids, but the details are unclear.

The recognition site of CIRP is a controversial issue in several reports. Nishiyama reported that GST-CIRP fusion protein binding to all RNA homopolymers to a varying extent at low NaCl concentrations, while at high NaCl concentrations, GST-CIRP fusion protein only binding to poly (U), suggesting that CIRP protein possesses a specific RNA-binding [12]. Yang R reported a different motif by computational analysis in 3′UTR in potential target mRNAs [30]. Our findings are partly in accord with Nishiyama's results. We found that CIRP could bind with 5′UTR and had a potential binding ability with the CR indicated that the recognition sites were not limited to the 3′UTR, which was supported by the studies by Zhang X [28] and Doukhanine E [44] in other RBPs. Our research focused on endogenous protein expressed in testis and analyzed normal RNA and fragments binding with CIRP by experiment procedures, validated the possible core recognition sequence, which support and improve Nishiyama's results. Although there are many differences between in vivo and in vitro binding analysis, we can make an inference that (Un)($n \geqslant 2$) is the most possible core recognition sequence of CIRP. CIRP may have a general binding ability to U-rich sequences which is strongly supported by so many target mRNAs presented on microarray assay and the results from Nishiyama's research. In addition, auxiliary recognition sequence may exist to enhance the binding ability. Secondary structure of RNA is ignored in the recognition sequence analysis nowadays [38,45–50] because there are no better research approaches to evaluate (see RBPDB database: http://rbpdb.ccbr.utoronto.ca/).

We performed microinjection in testis to deliver siRNA complex, and it was proved to be an efficient approach in RNAi research in testis. Microinjection has been used for stem cell transplantation and plasmid delivery [29,51–57] as described by Ogawa et al. in 1997. We discovered that siRNA delivered with Lipofectamine™ 2000 reagent to the testis in vivo is an effective method for RNA interference, and our recommended proportion of siRNA and Lipofectamine™ 2000 reagent is 1:3 (μg:μl). The appropriate volume of siRNA complex is 30 μl (for BALB/c mice testis: 100 mg), and the injection time should be 10 min or more.

In conclusion, CIRP may play important roles in the mouse testis and down-regulated CIRP in testis cause by heat stress may contribute to male infertility. We revealed that most of the target mRNAs of CIRP in testis were associated with translation regulator activity, antioxidant activity, envelope and reproduction. We found that the binding mRNAs increased their survival time significantly. We also validated that (Un)($n \geqslant 2$) is the most possible core recognition sequence of CIRP. More detailed functions of CIRP in testis required further researches, it very helpful to clarify the pathological mechanisms of infertility caused by heat stress.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2012.07.004.

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